

REMARKS

Applicants acknowledge, with appreciation, the courtesy extended to their representatives by the Examiner during a telephone interview on November 8, 2002. Applicants make that interview of record herein. As suggested by the Examiner, applicants request amendment of the claims to recite a computer further comprising a computer program.

Applicants request reconsideration of the above-identified application in view of the foregoing amendments and the following remarks.

Claims 39, 42 and 43 were pending in the present application at the time of the Office Action. Applicants request amendment of claims 39, 42 and 43 to place them in condition for allowance and appeal. Amended claims 39, 42 and 43 are supported on page 1, lines 1-5, page 33, lines 8-10, page 35, lines 29-32 and page 26, lines 14-21 of the specification. These amendments do not constitute new matter.

Applicants have also amended the specification such that cross reference to related applications to which the present application claims priority is added just beneath the title on page 1. No new matter is introduced.

I. Claim Rejections under 35 U.S.C. § 101

The prior rejections of claims 39 and 42 under 35 U.S.C. § 101 have been withdrawn on the basis that they are now directed to statutory subject matter. However, the Examiner maintains that “the structural coordinates are nonfunctional descriptive material and do not distinguish the invention in terms of patentability”².

² August 21, 2002 final Office Action, paper number 31, page 3, lines 2-3

More particularly, the Examiner contends that “the claims recite statutory subject matter as they are directed to an article of manufacture (i.e. a computer comprising a computer screen), not because they recite an interfunctional relationship between the computer and data stored therein”³. Applicants disagree with these contentions, and believe that they do not apply to amended claims 39, 42 and 43.

Each of amended claims 39, 42 and 43 recites a computer comprising a program for producing a three-dimensional representation of a binding site or molecule or molecular complex. Each of amended claims 39, 42 and 43 further recites that the computer comprises a computer-readable data storage medium comprising a data storage material encoded with computer-readable data, wherein the data comprises structure coordinates. By means of the computer program, that computer reads the coordinates from memory and upon recognizing each coordinate, causes a three dimensional representation to be constructed in a proper axis system. The computer program interrelates the computer and the structure coordinates. Therefore, the software and the structure coordinates form a functional relationship with the computers recited in the amended claims.

The specification enables and describes the computers recited in the amended claims. Molecular Graphics manipulations are done with QUANTA software run on a Silicon Graphics Indigo2 computer (the specification at page 35, lines 29-32). Computer programs for visualization and molecular modeling, such as QUANTA (the specification at page 35, lines 29-32; page 26, line 14-21), Sybyl (the specification at page 26, line 14-21) are listed in the specification to enable one of

³ August 21, 2002 final Office Action, paper number 31, page 3, lines 4-6

ordinary skill in the art to use a computer to produce and visually inspect a three dimensional representation and to use that representation for drug design.

Accordingly, amended claims 39, 42 and 43 “recite an interfunctional relationship between the computer and the data stored therein”⁴ and comply with 35 U.S.C. § 101.

II. Claim Rejections under 35 U.S.C. § 102

Claims 39, 42 and 43 stand rejected under 35 U.S.C. § 102(b) as being “anticipated by” Staley, Comp. Usage Mater. Educ. Proc. Symp., 113-122 (1985), abstract only (“Staley”). The Examiner contends that “where a claim recites nonfunctional descriptive material which is not functionally related to the substrate on which it is stored, the nonfunctional descriptive material is considered, but not entitled to patentable weight”⁵. For essentially the same reasons, claims 39, 42 and 43 stand rejected under 35 U.S.C. § 102(e) as being “anticipated by” United States patent 5,581,476 (“Osslund”).

As stated above, each of amended claims 39, 42 and 43 recites a computer for producing a three dimensional representation of a binding site for CD40 or molecule or molecular complex of CD40L. Each of the claimed computer comprises a computer program with instructions to produce a three dimensional representation, a computer-readable data storage medium comprising a data storage material encoded with computer-readable data, wherein the data comprises structure coordinates, and a computer screen for displaying a three dimensional representation. Contrary to the Examiner’s contention that the structure coordinates constitute non-

⁴ August 21, 2002 final Office Action, paper number 31, page 3, lines 5-6

⁵ August 21, 2002 final Office Action, paper number 31, page 4, lines 4-6

functional descriptive material, each of the computers of the amended claims comprises a computer program that functionally interrelates the computer and the structure coordinates (i.e., the software and the structure coordinates form a functional relationship with the computer).

Because the structure coordinates are in a functional relationship with the program and the computer, the amended claims are not anticipated by Staley or Osslund. Neither of those documents discloses a computer capable of producing any of the three dimensional representations recited in amended claims 39, 42 and 43. The Examiner contends that Staley and Osslund teach computers with memory and display screens “wherein the computer is capable of displaying a 3D representation of a molecule using crystallographic coordinates”⁶, thereby anticipating claims 39, 42 and 43 since the coordinates are nonfunctional descriptive material. As discussed above, the coordinates are not nonfunctional descriptive material as recited in amended claims 39, 42 and 43. Therefore, neither Staley nor Osslund anticipates the computers recited in those claims.

III. Claim Rejections under 35 U.S.C. § 103

Claims 39, 42 and 43 stand rejected under 35 U.S.C. § 103(a) as being “unpatentable over” Staley in view of Peitsch, et al., International Immunity, 5: 233-238 (1993) (“Peitsch”). The Examiner contends that Staley teaches a computer for three dimensional display of crystal structures while Peitsch teaches a display of a three dimensional representation of amino acid residues 115 to 260 of CD40L. The Examiner also contends that since the Peitsch coordinates have been deposited in the

⁶ August 21, 2002 final Office Action, paper number 31, page 4, lines 2-3 and lines 14-15

Protein Data Base, the structure is established. The Examiner further alleges that it would have been obvious to one skilled in the art at the time of the invention to display amino acid residues 115 to 260 of CD40L since the structure coordinates for CD40L were known. Applicants traverse, in view of the accompanying Declaration of Dr. Juswinder Singh under 37 C.F.R. § 1.132 (the “Singh Declaration”) and the following remarks. As demonstrated in the Singh Declaration, Peitsch neither teaches nor suggests the structure coordinates of human CD40L which characterize the three dimensional representation of the binding sites or molecule or molecular complex recited in amended claims 39, 42 and 43. Accordingly, use of the Peitsch model in Staley’s computer would not teach or suggest the computers of amended claims 39, 42 and 43.

The Singh Declaration presents experimental evidence obtained by comparing the Peitsch model to the crystal structure disclosed in the above-identified application which serves as the source for the structure coordinates recited in amended claims 39, 42 and 43. That evidence shows that the Peitsch model is not an accurate model for the structure of human CD40L. A backbone alignment between the structure of the binding site residues recited in amended claim 43 and the Peitsch model shows a root mean square deviation (rmsd) value of 2.8 Å (Singh Declaration, paragraph 10, lines 4-14). A backbone alignment between the structure of the entire CD40L crystal model of this application, as recited in claim 42, and the Peitsch model shows a root mean square deviation (rmsd) value of 2.88 Å (Singh Declaration, Figure 2 and paragraph 10, lines 15-18 and 22-25). An alignment between backbone atoms of residues Lys143, Tyr145, Arg203 and Arg207 of the crystal structure characterizing the three dimensional representation of a binding site of amended claim 39 and the

corresponding amino acid residues of the Peitsch model gives an rmsd value of 1.306 Å (Singh Declaration, paragraph 10, lines 15-22 and 25-28). These high rmsd values⁷ show that the Peitsch model is not an accurate and true model of human CD40L and, therefore, does not render obvious the structure coordinates recited in the amended claims.

Although Peitsch's model for amino acid residues 115 to 260 of CD40L has been deposited in the Protein Data Bank, that model was not generated by X-ray crystallography or NMR. Instead, Peitsch relates to a three dimensional model of mouse CD40L which was derived from molecular modeling and energy minimization based on the known crystal structure of human tumor necrosis factor α (TNF α). Mouse CD40L is only about 20-25% identical with human and mouse TNF α (23.2% and 25.7 %, respectively) (Peitsch at page 233, right column, first full paragraph, lines 13-14). Because Peitsch's mouse CD40L model is derived from human TNF α , with which it has little sequence similarity (Singh Declaration, paragraph 5, lines 2-4), that model is speculative, at best (Singh Declaration, paragraph 5, lines 4-7).

Moreover, the structure disclosed in this application is the structure of human CD40L (Singh Declaration, paragraph 6, lines 1-2 and the specification at page 1, lines 1-5). The human and mouse CD40L proteins share 78% homology (Singh Declaration, paragraph 6, lines 2-5). More importantly, the amino acid sequence of residues 115 to 260 of human CD40L which encompasses the binding site residues of

⁷ These rmsd values are above the rmsd cutoff value of 1.0 Å that the present application preferably requires of identical structures (see the specification, page 13, lines 16-23).

amended claim 43 for CD40 in human CD40L differ from the corresponding mouse CD40L residues (Singh Declaration, Figure 1 and paragraph 6, lines 5-9). For all these reasons, the Peitsch model must be recognized as a speculative model of mouse CD40L (Singh Declaration, paragraph 5, lines 4-7).

For the foregoing reasons, one of skill in the art would view the Peitsch model as no more than a speculative model for the structure of human CD40L. The experimental evidence provided by the Singh Declaration makes it clear that the Peitsch model is not an accurate model of human CD40L (Singh Declaration, paragraph 6, lines 9-10 and paragraph 7, lines 1-3) and is therefore unsuitable for the structure-based drug design for agonists or antagonists of human CD40L (Singh Declaration, paragraph 7, lines 1-5) achieved by computers of amended claims 39, 42 and 43.

Because Peitsch can not provide an accurate three dimensional representation of the binding site or molecule or molecular complex recited in amended claims 39, 42 and 43, Staley, viewed in combination with Peitsch, neither teaches nor discloses the computers of amended claims 39, 42 and 43.

Applicants request the Examiner consider and enter the foregoing amendments and pass this application to issue.

Respectfully submitted,

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APPENDIX A

Appendix A

39. (Thrice Amended) A computer for producing a three dimensional representation of a binding site for CD40 defined by structure coordinates of human CD40 ligand amino acids Lys143, Arg203, Arg207 and Tyr145, which correspond to residues 28, 88, 92 and 30, respectively, of SEQ ID NO: 3, according to Table 1;

wherein said computer comprises:

(a) a computer program with instructions to produce said three dimensional representation;

(b) a computer-readable data storage medium comprising a data storage material encoded with computer-readable data, wherein said data comprises the structure coordinates of human CD40 ligand amino acids Lys143, Arg203, Arg207 and Tyr145, which correspond to residues 28, 88, 92 and 30, respectively, of SEQ ID NO: 3, according to Table 1; and

[(b)] (c) a computer screen for displaying said three dimensional representation.

42. (Twice Amended) A computer for producing a three dimensional representation of a molecule or a molecular complex defined by the structure coordinates of all the human CD40 ligand amino acids according to Table 1; wherein said computer comprises:

(a) a computer program with instructions to produce said three dimensional representation;

(b) a computer-readable data storage medium comprising a data storage material encoded with computer-readable data, wherein said data comprises the structure coordinates of all the human CD40 ligand amino acids according to Table 1; and

[(b)] (c) a computer screen for displaying said three dimensional representation.

43. (Amended) A computer for producing a three dimensional representation of a binding site for CD40 defined by structure coordinates of human CD40 ligand amino acids Ile127, Ser128, Glu129, Ala130, Ser131, Thr135, Ser136, Ala141, Glu142, Lys143, Gly144, Tyr145, Tyr146, Cys178, Asn180, Ser185, Gln186, Ala187, Pro188, Ile190, Ala191, Ser192, Ser197, Pro198, Gly199, Arg200, Phe201, Glu202, Arg203, Ile204, Arg207, Ala209, Thr211, Pro217, Cys218, Gly219, Gln220, Glu230, Leu231, Gln232, Asn240, Val241, Thr242, Asp243, Ser245, Val247, Ser248, His249, Gly250, Thr251, Gly252 and Phe253, which correspond to residues 12, 13, 14, 15, 16, 20, 21, 26, 27, 28, 29, 30, 31, 63, 65, 70, 71, 72, 73, 75, 76, 77, 82, 83, 84, 85, 86, 87, 88, 89, 92, 94, 96, 102, 103, 104, 105, 115, 116, 117, 125, 126, 127, 128, 130, 132, 133, 134, 135, 136, 137, and 138, respectively, of SEQ ID NO: 3, according to Table 1; wherein said computer comprises:

(a) a computer program with instructions to produce said three dimensional representation;

(b) a computer-readable data storage medium comprising a data storage material encoded with computer-readable data, wherein said data comprises the

structure coordinates of human CD40 ligand amino acids Ile127, Ser128, Glu129, Ala130, Ser131, Thr135, Ser136, Ala141, Glu142, Lys143, Gly144, Tyr145, Tyr146, Cys178, Asn180, Ser185, Gln186, Ala187, Pro188, Ile190, Ala191, Ser192, Ser197, Pro198, Gly199, Arg200, Phe201, Glu202, Arg203, Ile204, Arg207, Ala209, Thr211, Pro217, Cys218, Gly219, Gln220, Glu230, Leu231, Gln232, Asn240, Val241, Thr242, Asp243, Ser245, Val247, Ser248, His249, Gly250, Thr251, Gly252 and Phe253, which correspond to residues 12, 13, 14, 15, 16, 20, 21, 26, 27, 28, 29, 30, 31, 63, 65, 70, 71, 72, 73, 75, 76, 77, 82, 83, 84, 85, 86, 87, 88, 89, 92, 94, 96, 102, 103, 104, 105, 115, 116, 117, 125, 126, 127, 128, 130, 132, 133, 134, 135, 136, 137, and 138, respectively, of SEQ ID NO: 3, according to Table 1; and

[b)] (c) a computer screen for displaying said three dimensional representation.

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39. (Thrice Amended) A computer for producing a three dimensional representation of a binding site for CD40 defined by structure coordinates of human CD40 ligand amino acids Lys143, Arg203, Arg207 and Tyr145, which correspond to residues 28, 88, 92 and 30, respectively, of SEQ ID NO: 3, according to Table 1;

wherein said computer comprises:

(a) a computer program with instructions to produce said three dimensional representation;

(b) a computer-readable data storage medium comprising a data storage material encoded with computer-readable data, wherein said data comprises the structure coordinates of human CD40 ligand amino acids Lys143, Arg203, Arg207 and Tyr145, which correspond to residues 28, 88, 92 and 30, respectively, of SEQ ID NO: 3, according to Table 1; and

(c) a computer screen for displaying said three dimensional representation.

42. (Twice Amended) A computer for producing a three dimensional representation of a molecule or a molecular complex defined by the structure coordinates of all the human CD40 ligand amino acids according to Table 1; wherein said computer comprises:

(a) a computer program with instructions to produce said three dimensional representation;

(b) a computer-readable data storage medium comprising a data storage material encoded with computer-readable data, wherein said data comprises the structure coordinates of all the human CD40 ligand amino acids according to Table 1; and

(c) a computer screen for displaying said three dimensional representation.

43. (Amended) A computer for producing a three dimensional representation of a binding site for CD40 defined by structure coordinates of human CD40 ligand amino acids Ile127, Ser128, Glu129, Ala130, Ser131, Thr135, Ser136, Ala141, Glu142, Lys143, Gly144, Tyr145, Tyr146, Cys178, Asn180, Ser185, Gln186, Ala187, Pro188, Ile190, Ala191, Ser192, Ser197, Pro198, Gly199, Arg200, Phe201, Glu202, Arg203, Ile204, Arg207, Ala209, Thr211, Pro217, Cys218, Gly219, Gln220, Glu230, Leu231, Gln232, Asn240, Val241, Thr242, Asp243, Ser245, Val247, Ser248, His249, Gly250, Thr251, Gly252 and Phe253, which correspond to residues 12, 13, 14, 15, 16, 20, 21, 26, 27, 28, 29, 30, 31, 63, 65, 70, 71, 72, 73, 75, 76, 77, 82, 83, 84, 85, 86, 87, 88, 89, 92, 94, 96, 102, 103, 104, 105, 115, 116, 117, 125, 126, 127, 128, 130, 132, 133, 134, 135, 136, 137, and 138, respectively, of SEQ ID NO: 3, according to Table 1; wherein said computer comprises:

(a) a computer program with instructions to produce said three dimensional representation;

(b) a computer-readable data storage medium comprising a data storage material encoded with computer-readable data, wherein said data comprises the structure coordinates of human CD40 ligand amino acids Ile127, Ser128, Glu129, Ala130, Ser131, Thr135, Ser136, Ala141, Glu142, Lys143, Gly144, Tyr145, Tyr146, Cys178, Asn180, Ser185, Gln186, Ala187, Pro188, Ile190, Ala191, Ser192, Ser197, Pro198, Gly199, Arg200, Phe201, Glu202, Arg203, Ile204, Arg207, Ala209, Thr211, Pro217, Cys218, Gly219, Gln220, Glu230, Leu231, Gln232, Asn240, Val241, Thr242, Asp243, Ser245, Val247, Ser248, His249, Gly250, Thr251, Gly252 and Phe253, which correspond to residues 12, 13, 14, 15, 16, 20, 21, 26, 27, 28, 29, 30, 31, 63, 65, 70, 71, 72, 73, 75, 76, 77, 82, 83, 84, 85, 86, 87, 88, 89, 92, 94, 96, 102, 103, 104, 105, 115, 116, 117, 125, 126, 127, 128, 130, 132, 133, 134, 135, 136, 137, and 138, respectively, of SEQ ID NO: 3, according to Table 1; and

(c) a computer screen for displaying said three dimensional representation.

APPENDIX B

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APPENDIX C

Recombinant Human CD40 Ligand Stimulates B Cell Proliferation and Immunoglobulin E Secretion

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Summary

Signaling through the cell surface molecule, CD40, is known to play an important role in the proliferation and differentiation of B lymphocytes. Using the thymoma cell line EL4, we recently identified and cloned a cDNA encoding a murine ligand for the CD40 molecule (mCD40-L) and showed that it has biological activity in vitro. A cDNA encoding a human homologue of the mCD40-L was isolated using crosshybridization techniques from an activated peripheral blood T cell library. The predicted amino acid sequence indicates that this human ligand for CD40 (hCD40-L) is a 261 amino acid type II membrane protein that exhibits 78% amino acid identity with its murine counterpart. Northern blot and FACS® analyses suggest that the hCD40-L is restricted in its expression to T lymphocytes, and that it is most abundant on the CD4⁺ T cell subpopulation. Cells transfected with hCD40-L caused the proliferation of human tonsil B cells in the absence of costimuli and, in the presence of interleukin 4, induced immunoglobulin E secretion from purified human B cells. A comparison of the efficacy of the hCD40-L and mCD40-L in these assays is presented.

CD40 is a cell surface protein expressed on B lymphocytes, follicular dendritic cells, normal epithelium, and some epithelial carcinomas. The predicted amino acid sequence for CD40 indicates that it is a member of the recently described TNF receptor family of proteins (1-3). This family includes such molecules as the low affinity receptor for nerve growth factor, both forms of TNF receptors, CD27, OX40, and the Hodgkin's lymphoma marker CD30 (3, 4). In addition to structural homology to known cytokine receptors, functional effects in B cells have been shown to be mediated through mAbs directed against CD40. These effects include short- and long-term proliferation (5-7), differentiation (8, 9), induction of intercellular adhesion (10, 11), and tyrosine phosphorylation of a series of intracellular proteins (12). Furthermore, germinal center centrocytes are prevented from undergoing apoptosis when activated through their antigen receptors and by CD40 mAb (13). Taken together, these data strongly suggested that CD40 was the receptor for an unknown ligand.

Recently, we reported the molecular cloning of a ligand for CD40 from the murine thymoma cell line EL-4, and showed that this ligand was biologically active on primary B lymphocytes (14). Cells transfected with mCD40-L could induce the proliferation of murine and human B cells in the absence of costimuli. In this paper, we detail the cloning of a human homologue of the ligand for CD40; describe the

relationship of the nucleotide and predicted amino acid sequences of the hCD40-L with those of the mCD40-L; examine the cellular distribution of the hCD40-L by Northern blot analysis and cell surface expression; compare the proliferative activity of both the hCD40-L and mCD40-L on human tonsillar and murine splenic B cells in the presence and absence of costimuli; and evaluate the ability of the two ligands to induce IgE secretion from IL-4-activated B cells.

Materials and Methods

Cell Separation. PBMC were purified from healthy donors by centrifugation over Histopaque® (Sigma Chemical Co., St. Louis, MO). Peripheral blood T cells (PB T)¹ were then purified by rosetting with 2-aminoethylisothiuronium bromide (AET)-treated SRBC and further centrifugation over Histopaque®. Contaminating monocytes were removed by plastic adherence for 1 h at 37°C. The resulting T cell preparations were always >98% CD3⁺, as determined by flow cytometric analysis (FACSscan®, Becton Dickinson & Co., Mountain View, CA). Tonsillar tissue was gently teased and the resulting cell suspension centrifuged over Histopaque®. T cells were purified as described for PB T cells. Purification of B cells was achieved by removal of cells rosetting with AET-treated SRBC and treatment of the remaining cells with B cell lympho-

¹ Abbreviations used in this paper: AET, aminoethylisothiuronium bromide; FBS, fetal bovine serum; PB T, peripheral blood T cells.

kwik (One Lambda Inc., Los Angeles, CA) for 1 h at 37°C to lyse contaminating non-B cells. The resultant B cell preparations were >96% CD20⁺ as determined by flow cytometry. Murine splenic B cells were isolated using anti-T cell antibody and complement, followed by passage over Sephadex G-10 (Pharmacia Fine Chemicals, Piscataway, NJ) to remove adherent cells. B cells were then positively selected by panning on plates coated with 5 µg/ml goat anti-mouse IgM (Southern Biotechnology Associates, Birmingham, AL). Isolated cells were >95% surface IgM⁺ as determined by flow cytometry.

Reagents. Murine and human recombinant IL-4 were purified from yeast supernatant as previously described (15). The 57-kD gene product of the extracellular domain of human CD40 fused to the Fc region of human IgG1 (CD40.Fc) was expressed, purified, and labeled with biotin-X-nonhydroxy succinimide (NHS; Calbiochem Corp., La Jolla, CA) as previously described (16). CV1/EBNA cells transfected with plasmids containing the hCD40-L or mCD40-L (see below) as described (14, 17), were cultured for 3 d on 10-cm petri dishes, removed and fixed with 1% paraformaldehyde for 10 min at 4°C, and washed extensively in culture medium before their use in biological assays.

Culture Conditions. For the preparation of RNA, PB T cells were activated for 20 h with immobilized CD3 mAb. Tonsil T cells were activated for 20 h with 10 ng/ml PMA and 1 µg/ml Con A. For proliferation assays, purified B cells (10⁵/well) were cultured in triplicate with a titration of CV1/EBNA cells expressing human or murine CD40 ligand in flat-bottomed 96-well microtiter plates in 200 µl RPMI in a humidified atmosphere of 10% CO₂. For the proliferation of murine B cells, medium was supplemented with 5% heat-inactivated fetal bovine serum (FBS), 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, and 50 µM 2-ME. For human B cells, medium was supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin. Wells were pulsed with 2 µCi [³H]thymidine (25 Ci/mmol) for the final 8 h of culture, cells were harvested, and incorporated cpm was determined by tritium-sensitive avalanche gas ionization detection on a beta counter (Matrix 96 Direct; Packard Instrument Co., Inc., Meriden, CT). Cultures for the determination of murine IgE secretion were performed as described for murine B cell proliferation assays but in supplemented RPMI 1640 containing 20% FBS. Culture conditions for the determination of human IgE were basically as described for human B cell proliferation assays but were conducted in round-bottomed 96-well microtiter plates. IgE levels in culture supernatants were determined by ELISA and were sensitive to 100 pg/ml and 4 ng/ml of human and murine IgE, respectively.

Flow Cytometric Analysis. Purified human PB T cells (10⁶/ml) were cultured with 10 µg/ml immobilized OKT3 (CD3, American Type Culture Collection) mAb in the presence or absence of 10 ng/ml PMA for the times indicated. Cells were harvested and preincubated with 100 µg/ml human IgG1 in PBS plus 0.02% NaN₃ for 30 min at 4°C, to prevent nonspecific binding of staining reagents. Surface expression of CD40-L was determined by staining with 5 µg/ml biotinylated CD40.Fc for 30 min at 4°C. Cells were washed twice with PBS plus 0.02% NaN₃ and incubated for 30 min longer at 4°C with streptavidin-PE diluted 1:5 in PBS plus 0.02% NaN₃ and then analyzed using a FACScan® (Becton Dickinson & Co.). A minimum of 5,000 cells were analyzed for each sample.

cDNA Library Construction. Pooled human PB T were isolated from normal donors and cultured in 10 ng/ml IL-2 for 6 d followed by stimulation with 10 ng/ml PMA and 1 µg/ml Con A for 8 h. Polyadenylated RNA was used as a template to construct a random

primed cDNA library using a cDNA synthesis kit (Amersham Corp., Arlington Heights, IL). cDNA was adapted using EcoRI linkers and cloned into a λgt11 vector (Stratagene Inc., La Jolla, CA). The library was screened with a ³²P-labeled mCD40-L riboprobe which was generated using SP6 polymerase (Promega Corp., Madison, WI) according to the supplier's protocol. This probe corresponds to the complete coding region of the mCD40-L gene, and was hybridized to the λgt11 library at 55°C in Stark's solution followed by washing in 2 × SSC 0.1% SDS at 63°C.

Transient Expression of hCD40-L in Mammalian Cells. The coding region of the hCD40-L was cloned into the mammalian expression vector pDC302 (17); DEAE transfections were performed in COS cells as described (18). Alternatively, pDC302 expression vector carrying the hCD40-L gene was cotransfected with the plasmid pSV3neo (19) using DEAE dextran into CV-1 EBNA cells (18).

Northern Blot Analysis. Northern blots were prepared essentially as described (20). Hybridization was performed in Stark's solution at 63°C followed by several washings in 0.2 × SSC, 1% SDS at 55°C. Blots were exposed for 1–3 d at –70°C.

Radiolabeling and Immunoprecipitations. COS cells expressing the hCD40-L protein or PB T cells cultured with immobilized CD3 as described for 3 h were radiolabeled with 100 µCi/ml ³⁵S Trans-label (ICN Biomedicals, Inc., Irvine, CA) for 2–3 h. Cells were lysed in PBS containing 1% Triton X-100 and clarified at 10,000 g for 30 min. Lysates were incubated with purified CD40.Fc protein (1 µg/ml) for 30 min followed by the addition of protein A-Sepharose. After extensive washing, pellets were resuspended in sample buffer in the presence or absence of β-ME and analyzed by PAGE.

Results

Characterization of the hCD40-L cDNA Clone. A ³²P-labeled riboprobe corresponding to the coding region of the mCD40-L was used to screen a stimulated human PB T library under reduced stringency (see Materials and Methods). This resulted in the isolation of a 1,803-bp cDNA that contained the complete coding region of the hCD40-L. This clone contained a single open reading frame that could encode a polypeptide of 261 amino acids, a 45-bp 5' noncoding region, and 975 bp of 3' noncoding sequences which included a poly(A) tail. The predicted amino acid sequence of the hCD40-L and an alignment with the CD40-L is shown in Fig. 1. Like the mCD40-L, the hCD40-L is a type II membrane protein with a short 22-amino acid NH₂-terminal cytoplasmic domain followed by a 24-amino acid hydrophobic signal anchor region. The hCD40-L has a 215-amino acid COOH-terminal extracellular domain compared to 214 amino acids in the mCD40-L and five cysteine residues compared to four in the murine ligand. There is an N-linked glycosylation site in the extracellular domain that is conserved between the human and murine ligands, and an additional, but likely not utilized, glycosylation site in the cytoplasmic domain of hCD40-L. The two sequences exhibit 78% amino acid identity overall. More specifically, there is 81% amino acid identity between the cytoplasmic domains, 96% between the transmembrane regions, and 75% between the extracellular domains.

Expression and Immunoprecipitation of Recombinant hCD40-L. Oligonucleotide primers designed to include incompatible restriction sites were used in a PCR reaction to amplify

	TGCCACCTTCTCTGCCAGAAGATACCACTTTCACTTTAACACAGCATGATCGAAACATAC	60
hCD40.L	M I E T Y	5
mCD40.L		
	AACCAAATCTCTCCCGATCTGCGGCCACTGGACTGCCATCAGCATGAAATTTTATG	120
hCD40.L	<u>N Q T</u> S P R S A A T G L P I S M K I F M	25
mCD40.L	S P V A	
	TATTTACTTACTGTTTTCTTATCACCCAGATGATGGGTGAGCACTTTTGTGTGTAT	180
hCD40.L	Y L L T V F L I T Q M I G S A L F A V Y	45
mCD40.L	V	
	CTTCATAGAAGGTTGGACAAGATAGAAGTGAAGGAATCTTCATGAAGATTTGTATTCT	240
hCD40.L	L H R R L D K I E D E R N L H E D F V F	65
mCD40.L	V E V	
	ATGAAAACGATACAGAGATGCAACACAGGAGAAAGATCCTTACTCTTACTGAACTGTGAG	300
hCD40.L	M K T I Q R C N T G E R S L S L L N C E	85
mCD40.L	I K L K K G	
	GAGATTAAAGCCAGTTTGAAGGCTTTGTGAAGGATATAATGTTAAACAAAGAGGAGCG	360
hCD40.L	E I K S Q F E G F V K D I M L N K E E T	105
mCD40.L	M R R D L T	
	AAGAAAGAAAACAGCTTTGAAATGCAAAAAGGTGATCAGAATCCTCAAATGGCGGCACAT	420
hCD40.L	K K E N S F E M Q K G D Q N P Q I A A H	125
mCD40.L	R E D	
	GTCATAAGTGAGGCCAGCAGTAAACAAACATCTGTGTACAGTGGGCTGAAAAGGATAC	480
hCD40.L	V I S E A S S K T T S V L Q W A E K G Y	145
mCD40.L	V N N A A K	
	TACACCATGAGCAACAACCTGGTAACCTGGAAAATGGGAAACAGCTGACCGTTAAAGA	540
hCD40.L	Y T M S N N L V T L E N G K Q L T V K R	165
mCD40.L	K S M	
	CAAGGACTCTATTATCTATGCCCCAAGTCACCTTCTGTTCCAATCGGGAAGCTTCGAGT	600
hCD40.L	Q G L Y Y I Y A Q V T F C S N R E A S S	185
mCD40.L	E V T P	
	CAAGCTCCATTATAGCCAGCCTCTGCCTAAAGTCCCCGGTAGATTGAGAGAATCTTA	660
hCD40.L	Q A P F I A S L C L K S P G R F E R I L	205
mCD40.L	R V G W P S I G S	
	CTCAGAGCTGCAATACCCACAGTTCGCCCAACCTTGGGGCAACATCCATTCACTTG	720
hCD40.L	L R A A N T H S S A K P C G Q Q S I H L	225
mCD40.L	K S Q L E V	
	GGAGGAGTATTGAATTGCAACCAGGTGCTTCGGTGTGTTGCAATGTGACTGATCCAAGC	780
hCD40.L	G G V F E L Q P G A S V F V <u>N V T</u> D P S	245
mCD40.L	A E A	
	CAAGTGAGCCATGGCACTGGCTTCACGTCCTTTGGCTTACTCAAACCTCTGAACAGTGTCA	840
hCD40.L	Q V S H G T G F T S F G L L K L *	261
mCD40.L	I R V S	
	CCTTGACGGCTGTGGTGGAGCTGACGCTGGGAGTCTTCATAATACAGCACAGCGGTTAAG	900
	CCCACCCCTGTTAACTGCCTATTATAACCCCTAGGATCCTCCTTATGGAGAATCTTTA	960
	TTATACACTCCAAGGCATGTAGAAGCTGTATAAGTGAATTACAGGTACATGAAACCAAA	1020
	ACGGGCCCTGCTCCATAAGAGCTTATATCTGAAGCAGCAACCCCACTGATGCAGACAT	1080
	CCAGAGAGTCTATGAAAGACAAGGCCATTATGCACAGGTGAATTCTGAGTAAACAGC	1140
	AGATAACTTGCCAAGTTCAGTCTTTGTTCTTTGCGTGCAGTGTCTTTCCATGGATAATGC	1200
	ATTGATTATCAGTGAAGATGCAGAAAGGAAATGGGAGCCTCAGCTCACATTCAGTTA	1260
	TGGTTGACTCTGGGTTCTATGGCCTTGTGGAGGGGGCCAGGCTCTAGAAGCTCTAACA	1320
	CAGTGGGAACCGAAACCCCGCCCGCCCTCTCGGACAGTTATTCAATCT	1380
	CTTTCAATCTCTCTCTCCATCTCTCTCTTTGAGTCTCTCTCTCAACCTCTTTCTTC	1440
	CAATCTCTCTCTCAATCTCTCTGTTTCCCTTTGTGAGTCTCTCTCCCTCCCGAGTCTC	1500
	TCTTCTCAATCCCTTTCTTAACACACACACACACACACACACACACACACACACACACA	1560
	CACACACACACAGAGTCAAGCGGTTGTAGTCAAGTCTCTCTTTTCCACCTGTCCCTAT	1620
	CTCTACCACTATAGATGAGGTGAGGAGTAGGAGTGCAGCCCTGAGCTGCCCACTCCT	1680
	CATTACGAAATGACTGTATTTAAAGGAAATCTATTGTATCTACCTGAGTCTCCATGTT	1740
	TCCAGAGTGAACCTGTAATTATCTTGTATTATTTTGAATGATACGACCTCTTAAC	1800
	ATT	1803

Figure 1. Nucleotide sequence exclusive of poly(A) of a cDNA encoding the hCD40-L protein (Genbank accession no. X67878). The predicted amino acid sequence of hCD40-L is given and compared with that of mCD40-L. Amino acid differences are indicated. The putative NH₂-terminal signal/anchor sequence is underlined, and potential N-linked glycosylation sites are shaded. A consensus sequence for addition of poly(A) is located at position 1784 (boxed), and a possible alternative poly(A) site occurs at position 989 (boxed).

the coding region of the hCD40-L gene. After digestion with the appropriate enzymes, the resulting PCR product was subcloned into a mammalian expression vector. Sequence analysis of the coding region insert confirmed that no nucleotide changes had occurred as a result of the PCR amplification. This plasmid was then used to transfect COS cells. The resulting recombinant hCD40-L was compared with the hCD40-L expressed on stimulated human PB T cells by metabolic labeling and precipitation using a previously described soluble CD40.Fc chimeric molecule (16). The results of this experiment are shown in Fig. 2. Immunoprecipitates electrophoresed under reducing conditions (lane b) indicate an apparent M_r of 33–35 kD for the recombinant hCD40-L.

This protein comigrates with the hCD40-L protein derived from CD3 mAb-stimulated PB T cells (lane d). Immunoprecipitates electrophoresed under nonreducing conditions are essentially indistinguishable (data not shown), suggesting that the hCD40-L does not dimerize through disulphide bonding. A larger band (~69 kD) is present in the samples from stimulated PB T cells (lane d) or cells transfected with the hCD40-L (lane b), but not in control samples (lanes c and e). We addressed the possibility that this band might be due to oligomerization of the ligand through noncovalent bond formation by electrophoresing precipitated samples on polyacrylamide gels containing 7 M urea in the presence and absence of reducing agents. When these experiments are performed,

the results are similar to those in Fig. 2 (data not shown). Thus, the exact nature of the higher molecular weight material is still unclear, but it is not likely to be a simple oligomer of the hCD40-L.

Characterization of hCD40-L Expression by Northern Blot Analysis. Previously, we reported that mCD40-L mRNA was induced in T cells activated with CD3 mAb (14). We examined purified human PB T and tonsil T cells for their expression of hCD40-L mRNA in response to various stimuli. Total RNA from PB T cells activated with CD3 mAb, or tonsil T cells stimulated with PMA and Con A, was analyzed on Northern blots using a ³²P-labeled antisense RNA probe (Fig. 3). No detectable hCD40-L was present in unstimulated PB T or tonsil T cell total RNA, but was inducible under both stimulation conditions. It is interesting that both PB T and tonsil T cells express what appear to be two hCD40-L-specific mRNAs. The larger band is typically more prominent than the smaller band and appears to be ~2,000 bases in length, comparable to the size of the single mCD40-L mRNA seen in murine T cells. The exact nature of the smaller band is unclear. PCR experiments (our unpublished data) have not provided any evidence for alternate mRNA splicing. There is only a single AATAAA polyadenylation consensus sequence in the 3' noncoding region (Fig. 1). However, the sequence AATAAG appears at position 989 and might possibly serve as an alternate polyadenylation signal.

Cellular Distribution of the hCD40-L. RNA was obtained from CD3 mAb-stimulated PB T cells and a number of cell lines of myeloid, lymphoid, fibroblastic, and epithelial origin, and examined for hCD40-L transcripts. The following cell types were found negative by Northern blot analysis using 5 µg poly(A)⁺ RNA per lane: Blin, CB23, Raji, Jurkat, PBM, monocytes, Thp-1, U937, HeLa, A549, placenta, and dermal fibroblasts. After hybridization with the ³²P-labeled antisense probe described above, no hCD40-L-specific mRNA could be detected in any cell type other than T cells or their derivatives. In addition, we examined a commercially available Northern blot containing total RNA derived from a variety of human tissues (Fig. 4). On this blot, the radiolabeled hCD40-L probe hybridized with RNA derived from lung tissue. This was somewhat surprising based on the

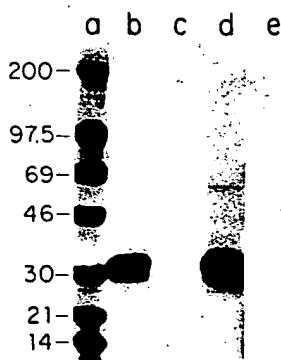


Figure 2. Precipitation of hCD40-L protein using soluble CD40.Fc. COS cells expressing the hCD40-L protein or CD3 mAb-stimulated PB T cells were radiolabeled as described in Materials and Methods. Lysates were precipitated using CD40.Fc and protein A-Sepharose and analyzed under reducing conditions on an 8-16% polyacrylamide SDS gradient gel. Lanes: (a) molecular weight markers; (b) recombinant hCD40-L protein derived from transfected cells; (c) cells transfected with empty vector; (d) hCD40-L protein from stimulated PB T cells; and (e) unstimulated PB T cells.

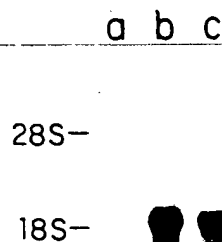


Figure 3. Northern blot analysis of hCD40-L-expressing T cells. Total RNA from stimulated PB T or tonsil T cells was analyzed on Northern blots using a ³²P-labeled antisense hCD40-L probe. Lanes: (a) unstimulated PB T cells; (b) CD3-stimulated PB T cells; and (c) PMA plus Con A-stimulated tonsil T cells.

Northern blot analysis of specific cell RNAs. However, it was possible that the hCD40-L-specific mRNA in this lung sample was due to contaminating T lymphocytes. To address this, we rehybridized this tissue blot with a ³²P-labeled antisense probe derived from the δ chain of the TCR (Fig. 4 B). This experiment resulted in the detection of a band of the expected size which was present exclusively in the lung-derived sample. Therefore, it appears that within the cell types examined thus far, expression of the hCD40-L is restricted to T lymphocytes.

Induction of hCD40-L on PB T Cells. The induction of hCD40-L expression on PB T cells was examined over 36 h after activation with CD3 antibody, PMA, or a combina-

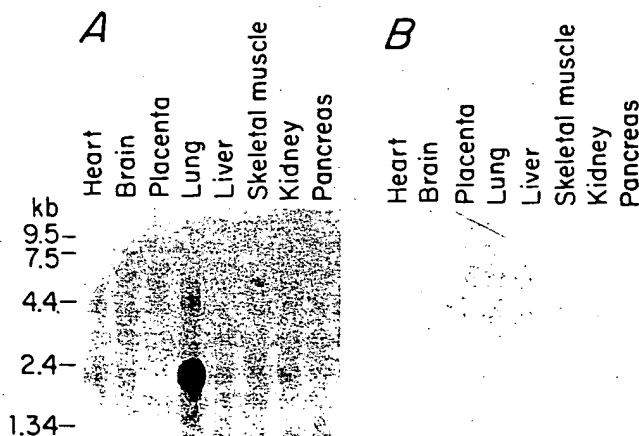


Figure 4. Northern blot analysis of tissue-specific RNAs. Poly(A)⁺ RNA from the indicated tissues was hybridized with antisense riboprobes as described in Materials and Methods. (A) Northern blot hybridized with a radiolabeled probe corresponding to the coding region of the hCD40-L. (B) The same Northern blot, partially stripped and rehybridized with a probe specific for the δ chain of the TCR.

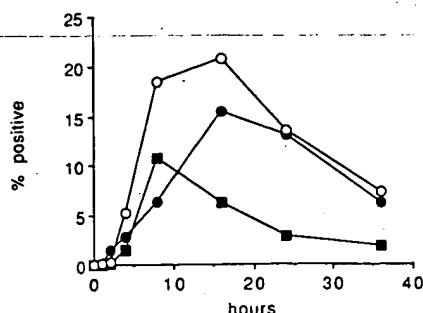


Figure 5. Induction of CD40 ligand expression on PB T cells. Purified peripheral blood T cells were activated for up to 36 h in the presence of 5 μ g/ml immobilized CD3 mAb (●), 5 ng/ml PMA (■), or a combination of CD3 mAb plus PMA (○). Cells were harvested at the time indicated, stained with 5 μ g/ml biotinylated CD40.Fc and streptavidin-PE, and analyzed by flow cytometry. Results are expressed as the percent of cells positive for biotinylated CD40.Fc binding compared to cells stained with control biotinylated IL-4R.Fc.

tion of the two (Fig. 5). Unstimulated T cells expressed virtually undetectable levels of hCD40-L, as determined by flow cytometric analysis of cells stained with biotinylated CD40.Fc. Treatment with CD3 mAb induced detectable hCD40-L by 4 h, with maximal expression at 8–16 h, and with significant expression still detected at 36 h. Stimulation with PMA resulted in lower levels of ligand expression but similar kinetics of induction. A combination of CD3 mAb and PMA induced detectable hCD40-L by 2 h and resulted in a higher level of ligand expression at 16 h than seen with either factor alone. In contrast to CD3 mAb, PHA alone was not an effective inducer of hCD40-L, but in combination with PMA resulted in levels of ligand comparable to those seen with a combination of CD3 mAb and PMA (data not shown).

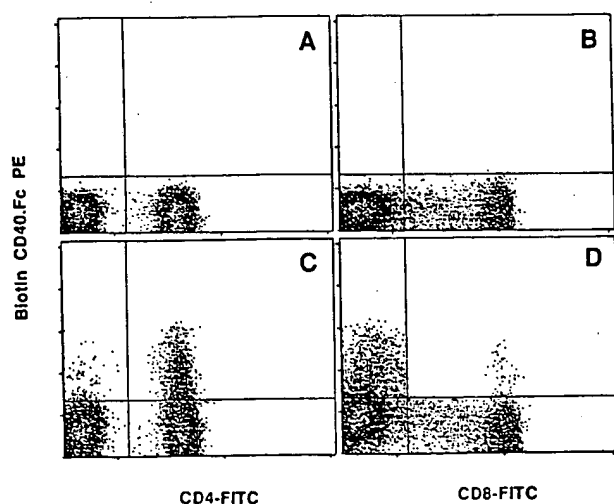


Figure 6. CD40 ligand is induced predominantly on CD4⁺ T cells. Purified PB T cells were cultured for 16 h in medium (A and B) or activated with 5 μ g/ml immobilized CD3 mAb (C and D) and stained with biotinylated CD40.Fc and streptavidin-PE and either FITC-conjugated CD4 (A and C) or CD8 (B and D) mAb. Results are representative from one of six experiments performed.

Expression of hCD40-L on T Cell Subpopulations. CD4⁺ and CD8⁺ PB T cells were examined for the expression of hCD40-L after 18-h stimulation with CD3 mAb (Fig. 6). In the absence of stimulation, CD4⁺ and CD8⁺ T cells expressed no detectable hCD40-L as determined by binding of biotinylated CD40.Fc (Fig. 6, A and B). After treatment with CD3 mAb, hCD40-L expression was detected primarily on the CD4⁺ population (Fig. 6 C). However, a reproducible finding from experiments performed on T cells from many donors was that a small proportion of CD8⁺ T cells was also induced to express hCD40-L (Fig. 6 D), although this expression was consistently lower than that seen on CD4⁺ cells.

Induction of Proliferation with CD40-L. The human and murine CD40-L were compared for their ability to mediate B cell proliferation. Purified human tonsil B cells were induced to proliferate in the absence of added cytokines when cultured with fixed CV1/EBNA cells transfected with either human or murine CD40-L (Fig. 7 A). This effect appeared to be dose dependent. Addition of IL-4 to cultures enhanced the proliferative response of human B cells to both human and murine ligand.

The effects of hCD40-L on proliferation of murine splenic B cells was also examined (Fig. 7 B). As shown previously, mCD40-L was mitogenic in a dose-dependent manner for murine B cells cultured in the absence of costimulus (14). In contrast, under the same culture conditions, CV1/EBNA cells expressing the hCD40-L had virtually no effect. However, the addition of IL-4 to cultures containing the hCD40-L resulted in the proliferation of murine B cells. Although IL-4

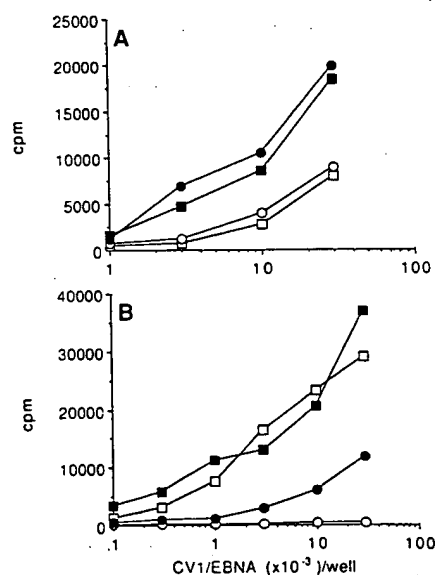


Figure 7. Comparative proliferative responses of B cells to human and murine CD40 ligand. (A) 10⁵ purified human tonsil B cells were cultured for 6 d with a titration of CV1/EBNA cells transfected with human (●, ○) or murine (■, □) CD40 ligand, in the presence (filled symbols) or absence (open symbols) of 5 ng/ml hIL-4. (B) 10⁵ purified murine splenic B cells were cultured for 3 d with CD40 ligand as described above in the presence or absence of 10 ng/ml mIL-4. Results are expressed as mean incorporated cpm from triplicate cultures.

enhanced the response of human B cells to mCD40-L (Fig. 7 A), no such enhancement was observed when IL-4 was added to murine B cell cultures containing mCD40-L (Fig. 7 B). In both human and murine proliferation assays, CV1/EBNA cells transfected with vector alone had no effect (13, data not shown).

The two forms of CD40-L were compared for their ability to induce IgE secretion from IL-4-activated B cells. Human and murine CD40-L showed comparable activity in the induction of human IgE secretion from purified tonsil B cells (Fig. 8 A). As was seen in the proliferation assays, this effect also appeared dose dependent. Similarly, both human and murine forms of CD40-L induced IgE from IL-4-stimulated murine splenic B cells (Fig. 7 B), although the human ligand appeared to exhibit lower activity than the murine ligand in this assay. No secreted human or murine IgE was detected in the absence of IL-4 or if CV1/EBNA cells transfected with vector alone were used (14, data not shown).

Discussion

Cell surface molecules expressed on activated T cells that provide contact-dependent help to B cells have been the subject of intense investigation. Recently, a molecule expressed on CD3-activated murine T cells that induces B cells to proliferate in the absence of a costimulus and stimulates IgE secretion from IL-4-activated B cells was cloned and identified as a murine ligand for CD40 (14). Using crosshybridization techniques, we have isolated a human ligand for CD40 that exhibits significant homology (78% identity at the amino

acid and 83% at the nucleotide levels) to its murine counterpart. This level of homology suggested that the hCD40-L would be active on both murine and human B cells in a manner similar to that described for mCD40-L (14). In terms of IgE secretion, this was indeed the case, although the hCD40-L displayed lower potency than the mCD40-L on murine B cells. However, with regard to induction of proliferation, we report significant differences between the murine and human CD40-L. Using murine B cells, hCD40-L requires IL-4 for induction of proliferation, whereas mCD40-L does not. On the other hand, in the human B cell culture system both hCD40-L and mCD40-L are capable of inducing significant proliferation in the absence of IL-4. Moreover, IL-4 augments the response of human B cells to hCD40-L and mCD40-L.

Experiments performed in our laboratory (unpublished data) indicate that human and murine B cells differ in the time at which the maximum proliferative response to either hCD40-L or mCD40-L is observed. In murine B cell cultures, the maximal proliferative response is seen at day 2, whereas human B cells show the highest rate of proliferation at day 5, regardless of whether hCD40-L or mCD40-L is used as a stimulus. Because hCD40-L and mCD40-L have comparable activity on human B cells, the inability of hCD40-L to induce the proliferation of murine B cells in the absence of IL-4 is intriguing. One possible explanation for this disparity could be a decreased affinity of hCD40-L compared to mCD40-L for murine CD40.

The predicted amino acid sequence for the murine CD40 molecule is 62% identical to the human CD40 (2). This level of homology might suggest that the binding of both mCD40-L and hCD40-L to either form of CD40 would be comparable. In support of this, we have previously shown that mCD40-L binds to human CD40 with high affinity (21). However, additional binding studies using hCD40-L and mCD40-L will be necessary to further define this interaction.

Another possible explanation for the disparity in the activity of the two ligands is that hCD40-L may not induce proliferation of murine B cells in the absence of costimulus unless the CD40 receptor molecule is being expressed at some required threshold level. It is known that CD40 is constitutively expressed on nonactivated human B cells at high levels relative to those observed on murine B cells (1, 2, 22, and our unpublished observations). The addition of IL-4 to activated murine B cells enhances CD40 mRNA expression (2), which could result in an increased surface expression sufficient to allow the observed proliferative effects. Human B cells also respond to IL-4 by upregulating CD40 expression. However, if a minimum number or relative density of CD40 is required for optimal signaling, the comparable activity of hCD40-L and mCD40-L on human B cells in the absence of IL-4 may simply reflect the increased level of CD40 expression. In contrast to the proliferation assays performed in the absence of a costimulus, the observation that hCD40-L and mCD40-L induce similar levels of IgE from murine B cells is likely due to the absolute requirement for IL-4 in secretion of this isotype.

The conformation of the ligands and the CD40 receptors no doubt play an important role in defining their relative

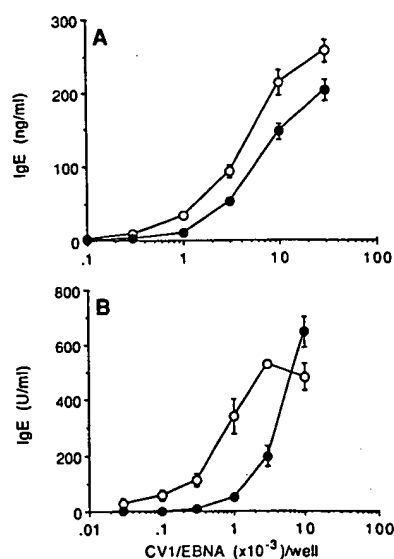


Figure 8. Human and murine IgE secretion induced by CD40 ligand. (A) 10^5 purified human tonsil B cells were cultured for 10 d with a titration of CV1/EBNA cells transfected with human (●) or murine (○) CD40 ligand, in the presence of 5 ng/ml hIL-4. (B) 10^5 purified murine splenic B cells were cultured for 7 d with CD40 ligand as described above in the presence of 10 ng/ml mIL-4. Results are expressed as mean values \pm SEM from triplicate cultures.

affinities for one another. Fine structure epitope mapping using panels of mAbs will be useful, however, as of yet, such reagents are unavailable. Recently, Lederman et al. (23) described a mAb directed against a 30-kD activated T cell surface antigen which resembles the hCD40-L in its functional effects. This antibody inhibits the B cell proliferation and CD23 expression induced by activated CD4⁺ T cells. However, the kinetics of induction of the T cell surface antigen recognized by this antibody differ from that seen in our laboratory for the hCD40-L. In addition, reactivity of this antibody is restricted to CD4⁺ T cells, whereas hCD40-L can also be induced on a small percentage of CD8⁺ T cells. Further studies will be necessary to determine the exact identity of the T cell antigen recognized by this antibody and its relationship to the hCD40-L.

The significance of the induction of the hCD40-L on a small proportion of CD8⁺ PB-T cells is unclear. Attempts to induce the expression of hCD40-L on human T cell clones (our unpublished observations) have thus far been successful only in CD4⁺ cells, despite the fact that several CD8⁺ clones have also been examined. Studies are currently underway to determine whether CD8⁺ cells expressing hCD40-L play a unique role in the normal immune response in vivo.

In addition to B cells, the cellular distribution of CD40 includes follicular dendritic cells and epithelial cells. Given the diversity of the response to B cells to the CD40-L, it will be of interest in the future to determine the role of the CD40-L in the biology of other cell types.

The authors wish to thank Steve Gimpel for sequencing the cDNA; Ken Grabstein and David Cosman for helpful discussions; and Anne C. Bannister for editorial assistance.

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Received for publication 30 July 1992.

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APPENDIX D

1cda: GDEDPQIAAHVVSEANSNAASVLQWAKKGYTMMKSNLVML
1aly: GDQNPQIAAHVISEASSKTTSVLQWAEKGYTMSNNLVTL

1cda: ENGKQLTVKREGLYYVYTQVTFCNREPSSQRPFI VGLWL
1aly: ENGKQLTVKRQGLYYIYAQVTFCNREASSQAPFIASLCL

1cda: KPSIGSERILLKAANTHSSSQLCEQQSVHLGGVFELQAGA
1aly: KSPGRFERILLRAANTHSSAKPCGQQSIHLGGVFELQPGA

1cda: SVFVNVTEASQVIHRVGFSSFGLLKL
1aly: SVFVNVTDPSPQVSHGTGFTSFGLLKL

Amino acid sequence alignment between amino acid residues Gly116 to Leu261 of the crystal structure model of human CD40L (1aly) in the present application and the corresponding amino acid residues in the Peitsch model of mouse CD40L (1cda).

APPENDIX E

The Structure of Human Lymphotoxin (Tumor Necrosis Factor- β) at 1.9-Å Resolution*

(Received for publication, August 16, 1991)

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The three-dimensional structure of recombinant human lymphotoxin (residues 24–171 of the mature protein) has been determined by x-ray crystallography at 1.9-Å resolution ($R_{\text{cryst}} = 0.215$ for $I > 3\sigma(I)$). Phases were derived by molecular replacement using tumor necrosis factor (TNF- α) as a search model.

Like TNF- α , lymphotoxin (LT) folds to form a “jellyroll” β -sheet sandwich. Three-fold related LT subunits form a trimer stabilized primarily by hydrophobic interactions. A cluster of 6 basic residues around the 3-fold axis may account for the acid lability of the trimer.

Although the structural cores of TNF- α and LT are similar, insertions and deletions relative to TNF- α occur in loops at the “top” of the LT trimer and significantly alter the local structure and the overall shape of the molecule. The structure of the “base” of the trimer is highly conserved. The sites of two mutations (Asp-50 and Tyr-108) that abolish the cytotoxicity of LT are contained within poorly ordered loops of polypeptide chain that flank the cleft between neighboring subunits at the base of the molecule, suggesting that the receptor recognizes an intersubunit binding site.

Lymphotoxin (LT,¹ also called tumor necrosis factor- β) is a lymphocyte-secreted cytokine 32% identical in primary sequence to monocyte/macrophage-derived tumor necrosis factor- α (TNF- α) (1, 2). LT exhibits pleiotropic activities very similar to those of TNF- α . Both molecules are cytotoxic to a variety of tumor cell lines *in vitro* and can induce hemorrhagic necrosis of tumors *in vivo* (3). Similarly, LT mediates proinflammatory (4) and antiviral (5) responses and alters the proliferative state of a variety of cell types (1). Both

TNF- α and LT bind to each of two distinct TNF receptors (6–8), but their relative affinities have not been well studied. In contrast, the cellular origins, mechanism of induction, and mode of secretion of LT and TNF- α are different (1), and the two cytokines produce different effects on several lymphoid, endothelial, and other cellular targets (9). Unlike human TNF- α , human LT is a glycoprotein, contains no disulfide bonds (3), and is unstable in the presence of acid, detergents, and organic solvents.

Because the three-dimensional structures of LT and TNF- α are key to understanding the differences in their biological and physical properties, we have undertaken crystallographic analysis of both molecules. We (10) and others (11) have reported the structure of TNF- α . Here we describe the 1.9-Å resolution structure of a truncated, active, recombinant, and non-glycosylated form of human lymphotoxin corresponding to residues 24–171 of the mature LT found *in vivo*.

EXPERIMENTAL PROCEDURES

Recombinant human lymphotoxin (residues 24–171) was expressed in *Escherichia coli* and purified as described previously (3). Crystals were grown by vapor diffusion at 4 °C from hanging drops containing 0.5 M NaCl, 20 mM Tris, pH 8.0, and 4 mg/ml protein over well solutions containing 40% saturated NaCl. Crystals of space group R32 (Table I) grew as thin hexagonal plates as large as 0.5 mm across and 0.08 mm thick.

Diffraction data were recorded from two native crystals using a Xuong/Hamlin-type multiwire area detector system (12). Graphite monochromated CuK α x-rays were produced by a Rigaku RU-200 generator operating at 5.4 kilowatts, and data were measured at 21–25 °C by omega scans at a speed of 0.1 degrees/min. “Shifts” of 5° of data were scaled and merged using the PROTEIN package (13). Individual scale factors were applied to each shift; a single exponential (B) scale factor was applied to all shifts from a single crystal.

Atomic coordinates for monomer “A” from data set 1TNF in the Brookhaven Protein Databank (10, 14) were used as a search model for phase determination by molecular replacement (15). Rotation and translation functions, and crystallographic refinement calculations were carried out using the program package X-PLOR (16).

Cross-rotation function solutions at 3.5 Å, eulerian angles $\theta_1 = 223.3$, $\theta_2 = 119.4$, $\theta_3 = 61.9$ (peak A) and $\theta_1 = 219.8$, $\theta_2 = 119.5$, $\theta_3 = 18.3$ (peak B) (related by a non-crystallographic 2-fold axis that was located in a native 3.5-Å Patterson self-rotation function at polar angles $\phi = 0.0$, $\psi = 22.5$, $K = 180.0$, 7.5° away from the hexagonal x axis in the xy plane) were refined by Patterson correlation (17). Peak A was the largest (3.8 S.D. above the mean) in a rotation function calculated over the asymmetric unit at 4.5-Å resolution using vector lengths from 5 to 25 Å and a 2.5° step size. Peak B was not among the 6000 highest peaks in this map but was located in a second rotation function calculated in 1.0° increments over a limited angular range ($\Phi_1 = 220.0 - 225.0^\circ$, $\Phi_2 = 115.0 - 122.5^\circ$, $\Phi_3 = 0.0 - 70.0^\circ$) using vectors from 15 to 25 Å in length.

Two $E_{\text{obs}}^2 E_{\text{calc}}^2$ translation function solutions (both 7 S.D. over the mean with data to 3.5-Å resolution), each corresponding to one of the two rotation function solutions, generated TNF-like trimers by crystallographic symmetry. Rigid body least squares minimization of $(F_{\text{calc}} - F_{\text{obs}})^2$ yielded a crystallographic R value of 0.51 for data to 3.5-Å resolution.

At this stage the model was rebuilt to reflect the amino acid sequence of LT (3). Model building and least squares superpositions were performed with the graphics program “O” (18). Five cycles of simulated annealing refinement (19) excluding non-crystallographic symmetry restraints after the first cycle, followed by manual inspection and rebuilding, were performed at resolutions of 2.5, 2.5, 2.25, 2.25, and finally 1.9 Å. Residues 24–32, 34–41, 39–46, 46–54, 84–91, 89–96, 99–103, 104–110, 110–117, 122–129, 158–165, and 168–171 were refit into simulated annealing omit maps (16) calculated at 2.25-

* Atomic coordinates have been submitted to the Protein Data Bank at Brookhaven National Laboratory for release 6 months after date of publication. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¹ The abbreviations used are: LT, lymphotoxin; TNF α , tumor necrosis factor- α .

TABLE I
Crystallographic data and refinement statistics

Crystal parameters	
Space group	R32
Cell constants (hexagonal setting)	$a = b = 55.86$ $c = 518.86$
Subunits/cell	36
Protein content	51%
Data collection statistics	
No. observations (all)	87,164
Observations, $I > 3\sigma_{(I)}$	52,136
No. unique reflections to 1.9 Å	23,666
R_{merge}^a	0.068
% complete at 1.9 Å	84.9
% complete 1.95–1.90 Å	67.5
Refinement statistics	
Number of atoms	2298
R_{cryst} (6–1.9 Å), $I > 3\sigma_{(I)}$	0.215
Root mean square deviation from ideal	
Bond lengths	0.014 Å
Bond angles	3.7°

^a $R_{\text{merge}} = \sum_h \sum_i |I_i(h) - I(h)| / \sum_h \sum_i I_i(h)$ where $I_i(h)$ and $I(h)$ are the i th and the mean measurement of the intensity of reflection h . $R_{\text{cryst}} = \sum_h ||F_{\text{obs}}(h) - F_{\text{calc}}(h)| / \sum_h F_{\text{obs}}(h)|$ where $F_{\text{obs}}(h)$ and $F_{\text{calc}}(h)$ are the observed and calculated structure factor amplitudes.

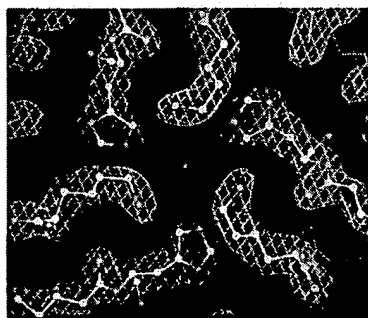


FIG. 1. A section of the $2F_{\text{obs}} - F_{\text{calc}}$ electron density map computed with data between 8 and 1.9 Å and phases determined from the refined atomic model and contoured at 1.5 σ units above the mean value of the map. The view is centered on and parallel to the crystallographic 3-fold axis and shows lysine 119 and histidine 131 of triad-related molecules A. The conformation about the His-131 C β -C γ bond could not be unambiguously determined.

Å resolution. Restrained, individual temperature factors were refined after the last cycle of simulated annealing. No solvent molecules are included in the present model.

Data collection and refinement statistics are summarized in Table I. A portion of the 1.9-Å electron density map is shown in Fig. 1.

Solvent accessible surface areas were computed with software written by T. Richmond based on the algorithm of Lee and Richards (28).

RESULTS AND DISCUSSION

Structure and Comparison to TNF- α —The tertiary fold of lymphotoxin is virtually identical to that of TNF- α (Fig. 2) (10, 11) and comprises a sandwich of two predominantly antiparallel β -pleated sheets of "jellyroll" Greek-key topology (20). The conformations of the β -sheets and of the loops connecting the sheets at the base of the LT subunit (Fig. 2) are very similar to those of the corresponding structures in TNF- α . Superposition of the two molecules using C α atoms of the β -strand residues only (Fig. 2) yields a root mean square positional deviation of 0.61 Å between the 108 equivalent atoms. On the basis of this superposition, the primary sequences of TNF- α and LT can be aligned as shown in Fig. 3.

The two crystallographically independent molecules of LT in the unit cell are quite similar within the β -sheet (root mean square positional deviation between molecules A and B after superposition of C α atoms in β -strands defined in Fig. 2 is 0.28 Å) but differ significantly in certain connecting loops. In molecule A the C-D (residues 84–94) and E-F (residues 123–127) loops are well ordered and stabilized by contacts with a symmetry-related molecule while in molecule B they are less well ordered and adopt different conformations. The conformation of the two molecules differs in other segments (A-A', residues 36–40; A'-A'', 48–51; D-E, 105–109) where the polypeptide chain is poorly ordered or poorly defined in the electron density.

Lymphotoxin subunits form trimers similar to those of TNF- α in which the 3-fold axis of the trimer is aligned approximately with the strands of the subunit β -strands. Trimers are assembled such that the "edge" of each subunit β -sandwich (strands E and F) is packed against the inner sheet "face" (strands A, A', H, C, and F) of its neighbor.

Of the 35 residues that form the hydrophobic core of the β -sandwich in LT, 20 are identical in TNF- α and only 5 are clearly non-conservative replacements. Several substitutions that might be expected to perturb the packing between opposing sheets are compensated by complementary changes in neighboring side chains. For example, the interacting pair of residues Phe-58/Ala-168 in LT are replaced by Val-41/Ile-154 in TNF- α , thus conserving hydrophobicity and volume. Substitution of Ser-117/His-135 in LT by Ala-96/Leu-120 in TNF- α replaces polar residues with a buried hydrophobic pair.

Only 13 of the 80 residues in the LT trimer with a solvent accessible surface area greater than 20 Å² are conserved in the primary sequence of TNF- α . The majority are located toward the base of the trimer in the polypeptide loops connecting the β -sheets (Fig. 2). The conserved Tyr-108 projects into the crevice between neighboring subunits.

The C-D loop in LT is 4 residues longer than that in TNF- α , while the E-F loop contains 5 fewer residues (Fig. 2). The longer C-D loop contains 1½ turns of helix and folds compactly across the top of molecule A but is extended and poorly ordered in molecule B. In TNF- α the C-D and E-F loops are connected by a disulfide bridge between Cys-69 and Cys-101. Both of these residues are substituted by tyrosine in human LT, and their C α atoms are separated by 13 Å. Although both cysteines are present in bovine and rabbit LT (21), they could not be expected to form a disulfide bond (Fig. 2). The C-D loop rolls away from the axis of the TNF trimer and, with the deletion in the E-F loop, creates a molecule that is less elongated than the TNF- α trimer and which flares open at the top (Fig. 5). The G-H loop in LT contains one fewer residue than that in TNF- α , but only local conformational differences are observed.

The lymphotoxin trimer is stabilized primarily by interactions between hydrophobic and aromatic side chains. Six aromatic residues and one histidine are present on the surface of the inner "face" sheet formed by strands A', A, H, C, and F (Fig. 4). Intervening glycine residues allow the aromatic residues to pack flat against the surface formed by the main chain atoms. This "aromatic tiling" is not as prominent in TNF- α , where the 3 phenylalanine residues nearest the base of the trimer are replaced by leucine or isoleucine. Three of the aromatic residues (Phe-74, Tyr-134, and Phe-169) pack around the trimer 3-fold axis.

At the top and bottom of the trimer, the subunit interface is characterized by charged and polar interactions. As in TNF- α , Lys-28 near the amino terminus forms a salt bridge with

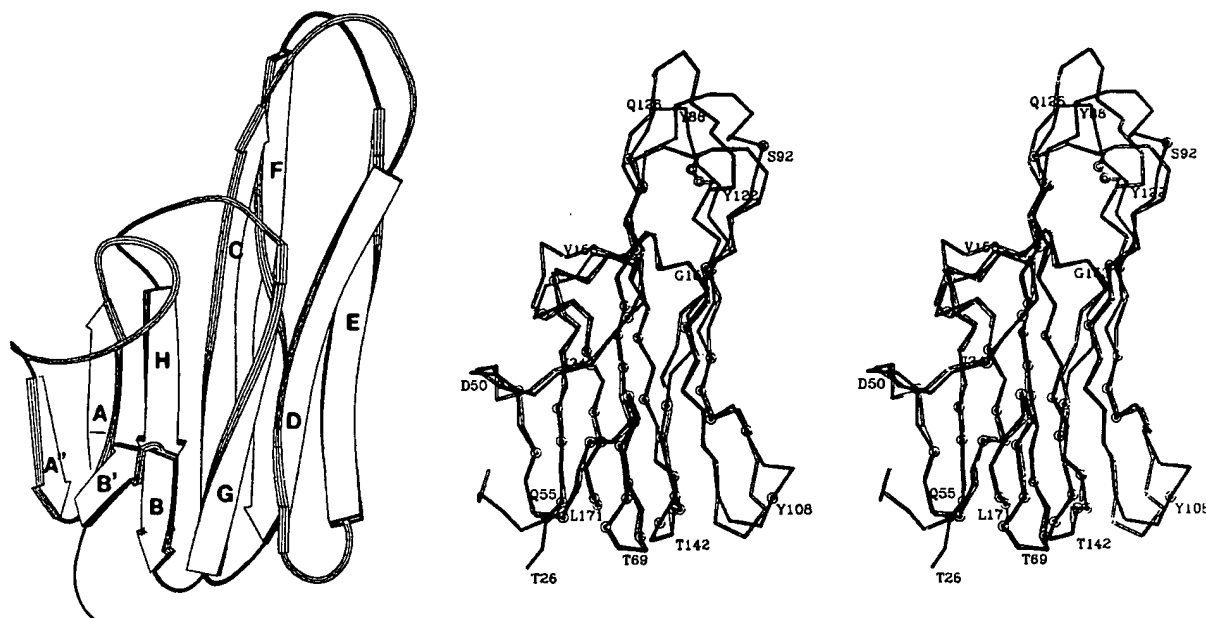


FIG. 2. Left, a ribbon tracing (27) the lymphotoxin monomer with β -strands labeled. They are, in strand order for the inner sheet facing the trimer axis: strand A', residues 52–56; A, 28–35; H, 164–169; C, 72–84; F, 127–140. Strands in the outer sheet are: A', 43–47 (not shown); B', 58–62; B, 64–68; G, 144–152; D, 93–105; E, 111–122. Right, least squares superposition of $\text{C}\alpha$ atoms of molecule A of lymphotoxin onto corresponding atoms of $\text{TNF-}\alpha$ (10) (molecule A of 1TNF from the Protein Databank (14)). Tyrosines 86 and 122 in LT correspond to cysteines 69 and 101 which form a disulfide bridge in $\text{TNF-}\alpha$. $\text{C}\alpha$ atoms shown with large spheres mark identically conserved residues in $\text{TNF-}\alpha$ and LT.

11	VRSSSRTP	19	SDKPVAVHVNPAEGQLQWLNRR	TNF
KP.AH....P....L.W....	Con
11	LPGLVLTSPAAQTARQHPKMLAHS	126	TLKPAHLIGDPSKQNSLLWRANTD	LT
134	NALLANGVELRDNLQVVPSEGLYLI	159	YSQVLFKGGGCP----STHVLTH	TNF
	..A.L..G..L..N.L.VP..G.Y..		YSQV.F.G.....S....L.H.	Con
	RAFLQDGFSLNNSLLVPTSGIYFV		YSQVVFSGKAYS PKATSSPLYLAHE	LT
151		176		
180	ISRIAVSYQTKVNLSSAIKSPCORE	105	TPEGAEAKPWYEPYLGGVFOLEK	TNF
Y...V.LLS..K.....	PW...Y.G..FOL..G	Con
	VQLFSSQYFPFVPLSSQKMYVP--		----GLQEPNLHSMYHGAAPQLTQ	LT
1101		1124		
1130	DRLSAEINRPDYLDFAESGQVYFGI	155	IAL	TNF
	D.LS.....L.....S..V.FG..		.AL	Con
	DQLSTHTDGIPLVLSPS-TVFFGA		FAL	LT
1145		1169		

FIG. 3. Primary sequence alignment of human LT (LT) and $\text{TNF-}\alpha$ (TNF) based on the best structural superposition determined in program "O" (18). Fifty of the 157 residues (32%) in $\text{TNF-}\alpha$ are identically conserved (Con) in lymphotoxin.

the carboxylate of the COOH terminus of the adjacent subunit, forming a ring of ion pair interactions around the 3-fold axis. Near the top of the trimer, Lys-119 and His-131 from each subunit form a cluster around the 3-fold axis (Fig. 1). The $\text{N}\epsilon$ atoms of Lys-119 are mutually separated by 5.4 Å but are located within 4 Å of the carbonyl oxygen of Leu-130 and the imidazole ring of His-131. Interactions with these groups and with a network of solvent molecules provide the only source of charge compensation for the lysine residues. On protonation, the histidine residues would contribute, together with lysines 119, six formal positive charges to the trimer interface, possibly accounting for the acid lability of LT. In contrast, in the more acid-stable $\text{TNF-}\alpha$, His-131 is replaced by a glutamic acid residue which forms a neutralizing ion pair with Lys-98, the counterpart to Lys-119 in LT.

Site of Receptor Binding—Goh *et al.* (22) have shown that

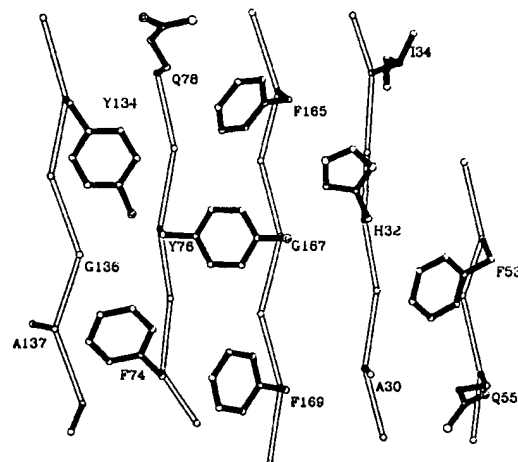


FIG. 4. The inner "face" of the LT monomer is tiled with aromatic residues. This surface packs against the "edge" of an adjacent subunit in the trimer, forming the hydrophobic core of the molecule. All side chains on this surface which project into the trimer interface are shown.

LT mutants with single amino acid substitutions at either Asp-50 or Tyr-108 have greatly reduced receptor binding and cytotoxic activity. These residues are contained within poorly ordered solvent-accessible loops (connecting strands A'-A' and D-E, respectively) that flank the interface between subunits in the trimer but do not participate in intersubunit contacts (Fig. 5). On the basis of this result and comparable results from mutagenic analysis of $\text{TNF-}\alpha$ (23, 24), it has been proposed (22, 23) that the receptor binds to $\text{TNF-}\alpha$ and LT in the cleft between subunits near the base of the trimer, which has been shown to be the active species (25). The flexibility of these loops in LT and $\text{TNF-}\alpha$ (10) might allow

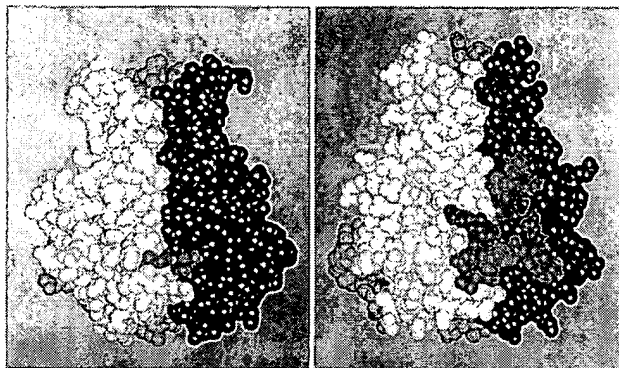


FIG. 5. van der Waals surface of the lymphotoxin (left) and TNF- α (right) trimers. Subunits are colored white, gray, and charcoal. Mutagenic "hot spots," associated with changes in cytotoxicity or receptor binding, are shown in red. On LT, these are Tyr-108 (on white subunit) and Asp-50 (on black subunit) (22). In TNF- α , mutagenesis of residues 84-91 (on white subunit), 30-34 (lower group on charcoal subunit), and 143-149 (upper group on charcoal subunit) can alter cytotoxicity (21, 23, 24).

structural adaptation to the binding surfaces of the two different receptors. The lack of sequence homology between TNF- α and LT in this region may indicate that the two cytokines employ alternative receptor binding conformations. Potentially, three receptors could simultaneously interact with a LT or TNF- α trimer, providing a mechanism for ligand-induced receptor aggregation (10, 21-23), which has been shown to mediate cytotoxicity in L-929 cells (26).

A region at the base of the TNF- α trimer, contiguous with the surface formed by the loops described above, has also been proposed to form part of the receptor binding surface (10). The structure and primary sequence of the A'-B; B'-B; B-C, and F-G loops are well conserved in TNF- α and LT. Conserved residues Pro-37, Gly-57, Gly-71, and Gly-144 maintain the conformation of the polypeptide turns, and leucines 54 and 60 could provide a hydrophobic receptor binding surface. Most mutations in these segments produce unstable, inactive molecules (24), which suggest that residues at these sites stabilize the tertiary or quaternary structure, but this does not exclude their involvement in receptor binding. A definitive description of LT-receptor interactions must await direct structural studies of the complexes.

Acknowledgments—We thank Dr. Anthony Kossiakoff for his continuing support of this project and Dr. Elizabeth Goldsmith for critically reading the manuscript.

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